Tumor Escape from Immune Recognition

Lethal Recurrent Melanoma in a Patient Associated with Downregulation of the Peptide Transporter Protein TAP-1 and Loss of Expression of the Immunodominant MART-1/Melan-A Antigen

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Abstract

In the last few years, mutiple protein target antigens for immunorecognition by T cells have been identified on human melanoma. How melanoma lesions escape from functional antigen-specific immune recognition remains poorly understood. We have identified the concomitant loss of the immunodominant T cell–defined MART-1/Melan-A antigen and downregulation of the TAP-1 gene in a recurrent metastatic melanoma that was resected in 1993. This phenotype was not observed for an earlier autologous melanoma lesion resected in 1987. The "antigen loss" could be restored in the variant tumor cell line by simultaneously providing both the MART-1/Melan-A gene (by retroviral transfer) and the TAP-1 gene (by a bioballistic approach) resulting in tumor cell sensitivity to MART-1/Melan-A–specific cytotoxic T lymphocytes. This suggests that tumor escape from immune surveillance may have occured in vivo as a sequential result of (*a*) antigen loss, and (*b*) downregulation of the peptide-transporter protein TAP-1 expression by this patient's tumor over a 6-yr period from 1987 to 1993. These results suggest that the characterization of the T cell response to melanoma in individual patients and definition of the immunologically relevant genetic defects in tumors may be required to select the most effective therapeutic strategies for a given patient. (*J. Clin. Invest.* 1996. 98:1633–1641.) Key words: tumor antigens • T cell recognition • TAP • human melanoma • antigen processing

Introduction

Melanoma is perhaps the tumor for which the best evidence exists for an effective immune response, with many tumor-bearing patients living for prolonged periods before succumbing to metastatic disease. It is also currently the tumor that has been most successfully treated with biologic response modifiers such as IL-2 and with the adoptive transfer of ex vivo–expanded patient T cells specific for tumors (1).

It is now generally accepted that T cells are the major immune effectors regulating tumor progression in an immunocompetent host. T cells derived from patients with cancers have been particularly useful in defining the molecular targets of immune reactivity, such as the melanoma-associated antigens MART-1/Melan-A (2–5), gp100/Pmel17 (6–8), or tyrosinase (9, 10). Recent advances in our understanding of tumor immunobiology have allowed for the identification of cellular mechanisms by which tumors may escape immune recognition (Table I) despite the presence of tumor-reactive T-lymphocytes in patients.

We describe a female patient diagnosed in 1976 with primary melanoma treated with multiple sequential surgical excisions. Tumor cells were available from resected lesions in 1987 and 1993. Cultured autologous HLA–A2+-restricted tumor-infiltrating lymphocytes (TIL)[1] were derived from the 1993 abdominal lesions. These TIL recognized not only the autologous tumor samples that had been resected in 1987 and 1993, but also other HLA–A2+-matched allogeneic melanomas. While tumor cells obtained from the 1987 lesion were recognized by a panel of allogeneic HLA–A2-restricted clonal and polyclonal cytotoxic T-lymphocytes (CTL) lines specific for recently identified CTL-defined antigens shared among melanoma cells and melanocytes (MART-1/Melan-A and gp100/Pmel17), the tumor cells resected in 1993 were not. These observations support the critical importance of T cell selective pressure against tumor progression and document two concurrent mechanisms of tumor evasion of immunity in vivo.

Methods

Cellular reagents. We describe in this report the characteristics of two melanoma lesions obained from a female patient diagnosed in 1976 with primary melanoma. She underwent primarily surgical resection of the melanoma lesion and subsequently presented with localized metastases. She was treated with various experimental therapies including monoclonal antibodies to p97 (11) and high dose IL-2. After a treatment response to IL-2, the patient subsequently progressed with isolated cutaneous and nodal metastases until 1993 when she relapsed, presenting with small bowel metastases, and ultimately succumbed to the disease. The melanoma cells examined included a freshly harvested single cell suspension (SCS), designated 717 from a lesion resected in 1987, an SCS (6129TW) obtained from resected small bowel lesions, and the derived melanoma cell line designated PM1-B1. Single cell suspensions from melanoma lesions and TIL were prepared as described in detail previously (12) and were stored

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1. *Abbreviations used in this paper:* CTL, cytotoxic T lymphocyte; ER, endoplasmic reticulum; FISH, fluorescence in situ hybridization; RT, reverse transcription; SCS, single cell suspension; TIL, tumor infiltrating lymphocyte.

Table I. Potential Defects in Melanoma Leading to Escape from Immune Recognition

Defect	Effects
Antigen loss or epitope loss in melanoma	Specific target of T cell recognition lost (29–31)
Point mutation within the MHC-presented T cell epitope	(I) Mutation abrogates binding to MHC molecules: target of T cell recognition lost (36). (II) Mutation does not affect binding to MHC: target of T cell recognition may be lost, or may function as 'partial agonist', or 'antagonist' leading to T cell anergy or to a qualitatively different effector T cell function(s) (33, 34).
Beta-2 microglobulin loss	Global defect in melanoma class I expression and CD8+ T cell recognition. MHC class II expression is intact (40, 43).
MHC class I global or allelic loss of MHC molecules	Global, individual decreased, or absent MHC class I expression on melanoma (such as HLA–A2, or B-locus alleles). Restricting MHC molecule for melanoma peptide presentation might be lost (40, 44, 45).
Peptide transporter defect	Decreased or absent expression of TAP-1. Failure in transporting peptides into the endoplasmic reticulum and expression of endogenously processed and presented MHC/peptide complexes on the cell surface. HLA–A2 expression is partially maintained due to binding of peptides derived from protein signal sequences in the ER (18, 19, 39, 40).
Downregulation of adhesion molecules, or costimulatory molecules	Delivery of signal 1 (MHC + peptide), but not signal 2 (costimulatory molecule) may induce T cell anergy. Loss of adhesion molecules may abrogate conjugate formation and consequently effector T cell functions (46).
Suppression of endothelial vascular adhesion molecule I (VCAM-1I) by melanoma	Downregulation of VCAM-1 prevents invasion of immune effector T cells to enter the tumor site (47).

in liquid nitrogen. Autologous TIL were maintained in AIM-V medium (GIBCO BRL, Grand Island, NY) supplemented with 1,000 IU IL-2 (provided by Cetus Corp., Emeryville, CA) in vitro without restimulation with tumor. Different effector CTL included the allogeneic polyclonal TIL line 1235, which has been previously used to identify the immunodominant HLA–A2-presented MART-1/Melan-A antigen (2–5), kindly provided by Dr. J.R.Yanelli (Surgery Branch, National Cancer Institute, Bethesda, MD). The HLA–A2-restricted CTL clones 1.1 and A83 have been described previously and exhibit similar specificity to that of TIL 1235, recognizing three closely related peptides provided by MART-1/MELAN-A (2, 5, 13). The CTL clone 2.37.1 recognizes a different set of melanoma peptides presented by HLA–A2 that are presumably derived from the gp100-Pmel17 protein. CTL targets included HLA–A2-matched melanoma cell lines and the LAK/NK sensitive target cell lines Daudi and K562 (obtained from Dr. Theresa Whiteside, IMD Laboratory at Pittsburgh Cancer Institute, Pittsburgh, PA). Recipient target cells in peptide pulsing assays included the human HLA–A2+ T/B cell hybrid cell line T2.

Cytogenetic analyses by fluorescence in situ hybridization (FISH). Metaphase cells were obtained from a melanoma cell line established from the melanoma single cell suspension resected in 1993 (called PM1-B1) using classical cytogenetic techniques (14). The chromosomes were trypsin-Giemsa banded. 20 metaphase cells were karyotyped and chromosomal abnormalities were expressed according to the International System for Cytogenetic Nomenclature (14). Cells labeled 717 (from the subcutaneous lesions resected in 1987), and 6129TW (from 1993) were melanoma cells, freshly dissociated after surgical excision and immediately cryopreserved in 10% DMSO in fetal bovine serum. The PM1-B1 line was initiated at the time of dissociation from a separate aliquot of the cell suspension that was labeled 6129TW and cryopreserved for later analysis. The cells were thawed, washed, and fixed in 3:1 methanol/acetic acid for 20 min at room temperature and slides were prepared. In preparation for FISH hybridization and analysis, the cell suspension from the PM1-B1 cell line (established from the 1993 lesion) and single cell suspensions 717 (1987), 6129TW (1993), and a control bone marrow specimen were applied to slides as described and treated according to the manufacturer's instructions (Oncor, Inc., Gaithersburg, MD). Pairs of satellite chromosome probes, one labeled with digoxigenin and the other with biotin for chromosomes 1 (D1Z5) and 3 (D3Z1), 1 and 7 (D7Z1), X (DXZ1), and 3, X, and 7, were prepared for hybridization. After hybridization, slides were washed and visualized using fluorescein-avidin/biotinylated antiavidin staining followed by rhodamine anti-digoxin antibody. The slides were counterstained with 4,6-diamidino-2-phenylindole (DAPI) in antifade solution and 250 interphase nuclei per pair were analyzed and photographed using a BHS microscope (Olympus Corp., Lake Success, NY) equipped with epifluorescence optics and a triple bandpass filter (digoxigenin/fluorescein/DAPI). Color photographs were taken on Ektochrome 100-HC color film (Eastman Kodak Co., Rochester, NY).

T cell "epitope mapping" of melanoma cell lines. MHC class I bound peptides were removed from viable tumor cells by mild acid elution leading to dissociation of MHC class heavy chains, beta-2 microglobulin, and peptides. Peptide material was fractionated by HPLC as previously described in detail (15, 16) and aliquots of individual HPLC fractions (containing peptide material) were pulsed on HLA–A2+ nonmelanoma antigen–presenting cells (i.e., the human T/B cell hybrid T2). Individual HPLC fractions were tested for T cell recognition in a standard 4-h ^{51}Cr release assay as described in detail (15, 16).

Reverse transcription–PCR analysis. Total RNA was prepared from tumor cells according to Chomczynski and Sacchi (17), reverse transcribed into cDNA, and tested for integrity using β-actin–speci-

fied primers. The primer for detection of the melanoma antigen MART-1/Melan-A has been reported previously (3), and the primer panel for genes involved in peptide transport (TAP1/2) and peptide generation (LMP2/7) has been described in detail elsewhere (18).

Immunostaining. The polyclonal rabbit antibody specific for the human TAP-1, but not TAP-2 (19) molecule was a kind gift of Dr. Hidde Ploegh (Massachusetts Institute of Technology, Cambridge MA) and was used for staining cytospins at a 1:1,000 dilution in PBS. Briefly, single-cell suspensions were prepared from the PM1-B1 line, and a single-cell suspension resected from 1987 (717) or from 1993 (6129TW) was blocked with nonimmune serum (1:10 in PBS), incubated for 5 h at room temperature with the anti–TAP-1 Ab, washed, incubated for 30 min with an $F(ab)'_2$ goat anti–rabbit Fc fragment, incubated for 30 min with ABC complex (Vectastain; Vector Laboratories, Inc., Burlington, CA), washed, incubated 9 min with aminoethylcarbozol (AEC; Biomeda, Foster City, CA), counterstained with hematoxylin, covered, and dried at 80°C.

Gene transfer. The human TAP-1 gene, cloned in the RSV5 vector was a generous gift from Dr. Thomas Spies, (Massachusetts Institute of Technology) and has been reported to restore TAP-1 function in human cell lines (20). The TAP-1 gene was delivered into melanoma cells by a bioballistic approach using TAP-1 DNA–coated gold particles that are accelerated by high air pressure to penetrate target cells. The acceleration device "gene gun" (Accell®) was provided by Agracetus Inc. (Middletown, WI). DNA coating of gold beads and acceleration was performed according to the manufacturer's instructions. The HLA–A2 gene was provided by Dr. R. Salter (Department of Pathology, University of Pittsburgh) and the full length (399 bp) human MART-1/Melan-A gene was cloned by reverse transcription (RT)-PCR using cDNA derived from the MART-1/Melan-A positive cell line Mel 624 and subcloned into the MFG-based proviral vector containing the selectable marker for G418 (geneticin) resistance (termed DFG-MART-1/Melan-A). Infectious retroviral supernatant was prepared as described in detail elsewhere (21) and the DFG-MART-1/Melan-A–infected melanoma line PM1-B1 was selected for 3 wk in CMRL medium (GIBCO BRL), supplemented with 400 μg/ml G418 (Gibco Laboratories, Grand Island, NY).

Results

TIL–PM1-B1 (1993) recognizes a subdominant T cell epitope on the autologous tumor. TIL derived from patient PM1-B1 in 1993 were grown in vitro for 4 wk, without restimulation with autologous or allogeneic tumor cells. TIL exhibited HLA–A2-restricted lysis of tumor targets in a standard 4-h ^{51}Cr release assay (data not shown). For a more detailed analysis of TIL specificity, MHC class 1 bound peptides were acid eluted from the autologous tumor cell line PM1-B1 or the

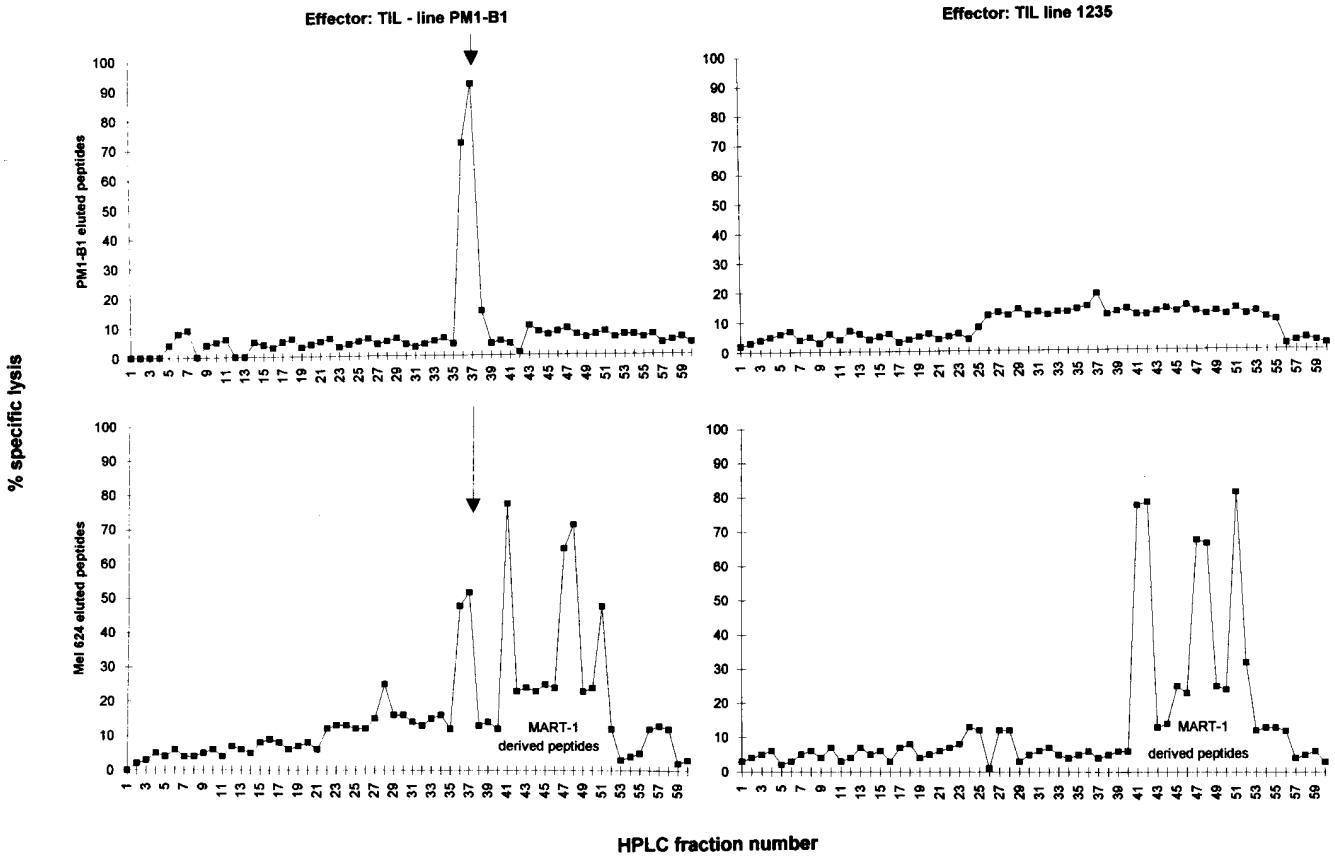

Figure 1. T cell epitope mapping: MART-1/Melan-A–derived T cell epitopes are absent in PM1-B1 (1993). Peptides were eluted from either the tumor cell line PM1-B1 or the HLA–A2+ control melanoma 624, and HPLC fractionated. Individual HPLC fractions were pulsed on the HLA–A2+-recipient target cell line T2 and tested for T cell recognition by cytotoxicity using either the autologous TIL line PM1-B1, or the MART-1/Melan-A–specific TIL line 1235 as effector T cells. TIL 1235 fails to detect MART-1/Melan-A–provided epitopes on PM1-B1, which are present on the control Mel line 624 eluting in HPLC fractions 41/42, 47/48, and 51. The TIL line PM1-B1 also recognizes such MART-1/Melan-A–derived bioactive HPLC fractions using peptides from the allogeneic Mel line 624. Such peptides are absent on the autologous tumor PM1-B1. Additionally, the TIL PM1-B1 recognizes a different HLA–A2-presented peptide(s) eluting in HPLC fraction 37 from the autologous tumor, and from the HLA–A2+ allogeneic Mel 624 (*arrow*).

HLA–A2+-matched allogeneic melanoma cell line 624, and HPLC fractionated. Aliquots from each individual HPLC fraction were pulsed onto HLA–A2 nonmelanoma targets (i.e., T2) and tested for T cell–mediated cytotoxicity using the autologous TIL line PM1-B1 or the MART-1/Melan-A–specific TIL line 1235 as effector cells (Fig. 1). TIL line PM1-B1 recognizes only a single dominant bioactive peptide peak eluting in HPLC fraction 37 (82% specific lysis) when peptides from the autologous tumor PM1-B1 were pulsed onto the HLA–A2+ target cell line T2. This target peptide had not been previously identified as a melanoma TIL epitope in our previous series of other HLA–A2+ melanomas, but a careful retrospective analysis of all available data suggests that this bioactive HPLC fraction contains a "subdominant" peptide epitope recognized by freshly harvested TIL (13, 16), but not by long-term cultured TIL. In marked contrast, TIL line PM1-B1 recognizes four bioactive HPLC peaks, containing Mel 624 cell-derived peptides, eluting in the corresponding HPLC fraction 37, but also three additional peaks eluting in fractions 41/42, 47/48, and 51. The MART-1/Melan-A–specific CTL line TIL 1235 did not recognize any bioactive peptide material eluted from Mel PM1-B1, but recognized three bioactive HPLC peaks eluting in HPLC fractions 41/42, 47/48, and 51, which contain T cell epitopes eluted from Mel 624 cells pulsed onto T2 cells. The bioactive HPLC peaks eluting in HPLC fractions 41/42, 47/48, and 51 appear to contain three closely related peptides

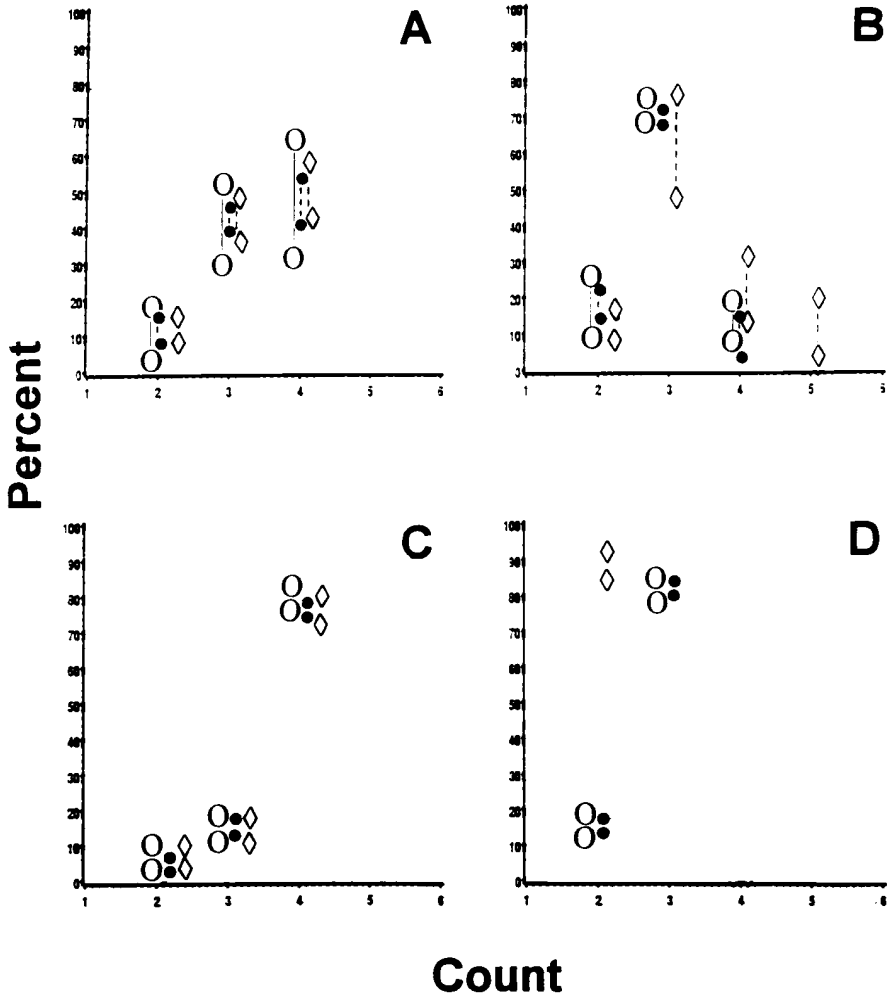

Figure 2. Similar cytogenetics of two different tumor cell preparations obtained in 1987 and 1993: graphic illustration of similarities in copy of chromosomes 1, 3, 7, and X in the two directly harvested cell suspensions, 6129TW (○), 717 (●), and the cell line PM-B1 (◇). The tumor cell line PM1-B1 was established from an SCS resected in 1993 (6129TW). We could not establish a melanoma cell line from the melanoma specimen obtained in 1987, hence only the SCS (717, 1987) was available for subsequent immunologic testing. To evaluate (*a*) similarities or gross changes in chromosome numbers between the tumor specimens obtained between 1987 and 1993, and (*b*) if the melanoma cell line PM1-B1 is representative of the SCS 6129TW (1993), we performed FISH analysis for the copy number of chromosomes 1, 3, 7, and X in the cultured PM1-B1 cell line, and the freshly dissociated and cryopreserved (> 97% tumor cells) SCS 717 and 6129TW. The graphs show the results of two independent determinations of chromosome copy number frequency. Pairs of satellite chromosome probes, one labeled with digoxigenin and the other with biotin (pairs 1+3, 1+7, X+3, and X+7) were hybridized to slides and counterstained with 4,6-diamid-ino-2-phenylindole as described in Methods. 250 interphase nuclei per pair were analyzed and photographed. The individual chromosome counts are presented as follows: (*A*) chromosome 1; (*B*) chromosome 3; (*C*) chromosome 7; and (*D*) chromosome X. The counts for individual satellite chromosome probes showed constant results in two independent experiments. For instance, the symbols for the individual cell populations examined (717 SCS [1987], *closed circles*, 6129TW SCS [1993], *open circles*, and the 1993 established cell line PM1-B1, (*diamonds*) are very close together in *C* and *D*, exhibiting almost identical counts in two independent experiments. However, we observed limited variation (*A* and *B*) for the chromomsome 1– and 3–specific probes indicated by bars or dotted lines. For instance in one experiment, examination of 250 interphase nuclei yielded 60% of cells showing four copy numbers of chromosome 1 for the cell line PM1-B1 (*A*, *diamonds*), and yet in the repeat, 44% of cells staining for chromosome 1. These data indicated that (*a*) three to four subpopulations are present within the freshly harvested tumor cell lines and within the cultured cell line PM1-B1 as defined by chromosome copy numbers of chromosomes 1, 3, 7, and X; (*b*) the freshly harvested SCS obtained from resected lesions in 1987 and 1993 appear to be closely related as regards the copy numbers of chromosomes 1, 3, 7, and X; and (*c*) the melanoma cell line PM1-B1 resembles (as regards the chromosome markers examined) the freshly harvested SCS obtained from the resected 1993 lesion. In general, the FISH data strongly suggested that copy numbers of chromosomes 1, 3, and 7 remained constant between all three cell populations examined. Note that in *D*, only the cell line PM1-B1 (*diamonds*) exhibits 90–94% of cells with two X chromosomes. In contrast, the freshly harvested tumor SCS from 1987 and 1993 (*open* and *closed circles*) exhibit 80–88% of cells with three X chromosomes, indicating alteration of the cell line PM1-B1 during in vitro cell culture.

Table II. T cell Epitopes Derived from MART-1/Melan-A Are Absent on the Tumor Resected in 1993, but Are Present on the Tumor Resected in 1987

		Mel-line 614 (control)		Mel line PM1-B1 (1993)		SCS 6129 (1993)		SCS 717 (1987)	
Effector TIL	Target antigen	Control IgG	W6/32	Control IgG	W6/32	Control IgG	W6/32	Control IgG	W6/32
E:T = 10:1		*Percent specific lysis*							
PM1-B1		50	0	20	0	13	0	50	0
TIL 1235	MART-1/Melan-A	37	1	2	1	0	0	28	2
TIL 1.1	MART-1/Melan-A	31	0	1	0	0	0	38	0
TIL A83	MART-1 Melan-A	27	2	0	0	0	0	20	0
TIL 2.371	gp100	18	1	1	0	0	0	16	0

To evaluate MHC class I–presented T cell epitopes on the control tumor Mel 624, the single cell suspension harvested in 1987 (SCS 17), the SCS harvested in 1993 (SCS 6129TW), and its derivative, the tumor cell line PM1-B1, were assayed for T cell recognition in a standard 4-h ^{51}Cr release assay. Specific T cell recognition was tested by blocking with an antibody directed against MHC class I (W6/32), or with control IgG. Four different CTL lines failed to kill the melanoma resected in 1993, but killed the tumor resected in 1987. Only the autologous TIL line PM1-B1 recognizes the tumor resected in 1993 (20% lysis).

provided by the MART-1/Melan-A antigen (2–5), which is expressed in melanoma cells and in normal melanocytes (2–5, 13, 22). Based on these T cell epitope–mapping experiments, it is suggested that the TIL present in the melanoma lesion resected in 1993 (PM1-B1) recognize MART-1/Melan-A–provided peptides, which are also recognized by the MART-1/Melan-A–specific allogeneic TIL line 1235. TIL PM1-B1 additionally recognizes an HLA–A2-presented melanoma T cell epitope eluting in HPLC fraction 37 that is expressed by both autologous and on HLA–A2-matched allogeneic melanoma cells. The sequence identity of its protein precursor is currently the focus of intensive study.

T cell epitopes defined by CTL clones are present on PM1-B1 (1987) but have been lost from PM1-B1 (1993). To compare the expression of T cell epitopes on the melanoma lesion resected in 1987 with the melanoma lesion resected in 1993, we tested several anti–MART-1/Melan-A or anti–gp100/Pmel17 CTL clones against a control target (Mel 624 cells), the freshly harvested SCS 717 (1987), SCS 6129TW (1993), and the melanoma cell line PM1-B1 (1993). Classical cytogenetic and FISH analysis for chromosomes X, 1, 3, and 7 revealed that the melanoma specimens resected in 1987 and 1993 are very closely related and that the melanoma cell line PM1-B1 is representative of the freshly isolated tumor cells harvested in 1993 (Fig. 2). TIL clones 1.1 and A83 are HLA–A2-restricted CD8+ CTL clones recognizing three closely related peptides provided by the melanoma antigen MART-1/Melan-A (2–5, 13, 22). The TIL clone 2.37.1 recognizes a different set of HLA–A2-presented melanoma peptides, eluting in HPLC fractions 27 and 33, presumably provided by the melanoma antigen gp100/Pmel17. Additionally, TIL PM1-B1 was evaluated for its ability to react against a panel of tumor target cell lines. All four TIL lines exhibited MHC class I–restricted lysis of the HLA–A2 control melanoma 624, and the SCS 717 resected in 1987 (Table II). In contrast, only the (autologous) TIL line PM1-B1 lysed the SCS 6129TW (1993) and its derived tumor cell line PM1-B1 (1993). MART-1/Melan-A–specific TIL clones A83 and 1.1, and the gp100-reactive TIL clone 2.37.1,

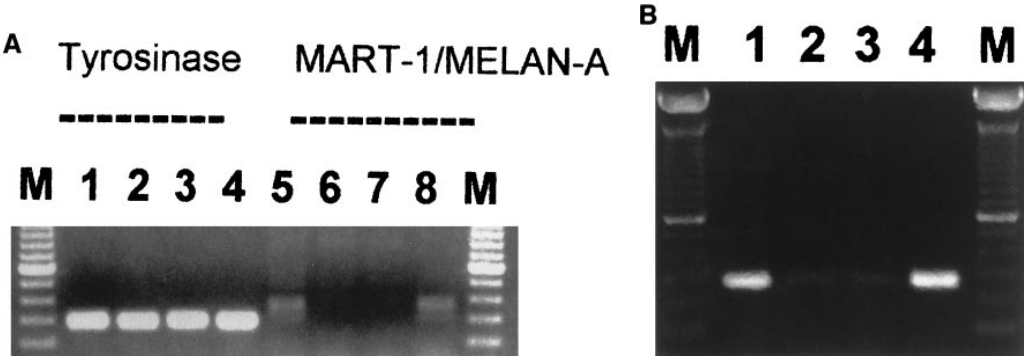

Figure 3. MART-1/Melan-A mRNA is absent in the melanoma resected in 1993 but present in 1987. (*A*) RNA was extracted, reverse transcribed into cDNA and tested for expression of tyrosinase, or MART-1/Melan-A by RT-PCR. (Lanes *1* and *5*) SCS 717 (1987), (Lanes *2* and *6*) SCS 6129TW (1993), (Lanes *3* and *7*) the derived Mel line PM1-B1 (1993), (Lanes *4* and *8*) control Mel 624. Primers employed to detect MART-1/Melan-A covered the entire gene product (399 bp). In summary, tyrosinase mRNA could be detected in the freshly harvested tumor cells obtained in 1987 (lane *1*) and 1993 (lane *2*), in the 1993 established cell line PM1-B1 (lane *3*), as well as in the positive control melanoma cell line 624 (lane *4*). In contrast, MART-1/Melan-A mRNA could be detected in the freshly harvested tumor cells from 1987 (lane *5*) and in the positive control melanoma cell line 624 (lane *8*). Freshly harvested tumor cells from the 1993 resected melanoma lesion (lane *6*) and the derived melanoma cell line PM1-B1 (lane *7*) tested negative for MART-1/Melan-A mRNA expression. A similar result was obtained using MART-1/Melan-A–specific primers spanning exons 2 and 5 (*B*). Freshly harvested tumor cells from 1987 (lane *1*) and the control melanoma 624 (lane *4*) showed MART-1/Melan-A expression. In contrast, freshly harvested tumor cells from 1993 (lane *2*), or the derived melanoma cell lines (lane *3*) showed low, or absent, MART-1/Melan-A mRNA expression.

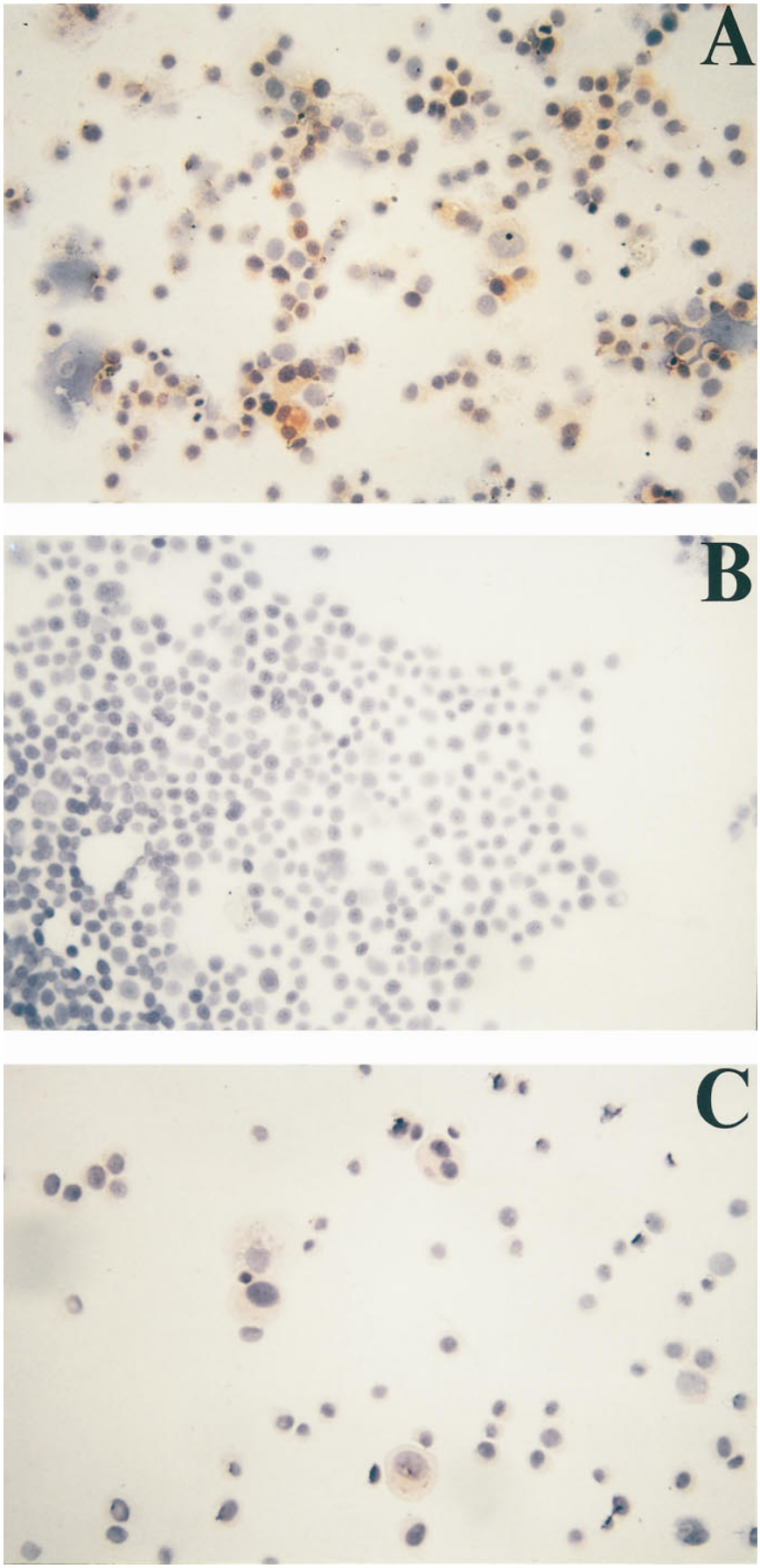

Figure 4. The peptide transporter molecule TAP-1 is expressed in the melanoma resected in 1987, but not in 1993. Cytospins were prepared and stained for TAP-1 protein expression (showing red staining) using an antibody specific for the TAP-1 protein. Melanoma cells resected in 1987 stained postive for TAP-1 (*A*). In contrast, TAP-1 protein is absent in the melanoma resected in 1993 (*B*), but could partially be restored (*C*) using the expresssion plasmid RSV5-TAP1 and the gene-delivery device Accell®.

failed to recognize 6129TW, or the melanoma line PM1-B1 (1993), confirming the absence of MART-1/Melan-A–derived T cell–defined epitopes presented on the tumor resected in 1993, but not in 1987.

Detection of antigen loss: MART-1/Melan-A mRNA is expressed by PM1-B1 (1987), but not by PM1-B1 (1993). RNA was extracted from the melanoma cells and reverse transcribed into cDNA to test for the presence of the MART-1/Melan-A mRNA. As a control, the message for tyrosinase, a key enzyme of melanin formation, was also evaluated (Fig. 3, *A* and *B*). All samples (717, SCS 1987; 6129TW, SCS 1993; Mel line, PM1-B1 1993; and the control, melanoma 624) exhibited a bright signal for tyrosinase. In contrast, only sample 717 (SCS, 1987) and the control melanoma 624 showed a positive signal for MART-1/Melan-A. Neither the freshly harvested SCS (1993) (6129TW) nor its derived cell line PM1-B1 (1993) expressed mRNA for MART-1/Melan-A.

Gene transfer of MART-1/Melan-A and TAP-1 into PM1-B1 (1993) restores the expression of MART-1/Melan-A epitopes recognized by CD8+ cytotoxic T cells. The MART-1/Melan-A gene was cloned from cDNA derived from (control) melanoma cell line 624. A retroviral vector with a selectable marker (Geneticin) termed DFG-MART-1/Melan-A was constructed for retroviral gene transfer of the MART-1/Melan-A gene. Examination of the antigen processing (proteasome subunits LMP2/LMP7) and presentation machinery (the peptide transporter heterodimer TAP1/TAP2) by RT-PCR analysis has revealed that TAP1 mRNA expression appeared to be downregulated in the melanoma lesion resected in 1993 compared with the tumor lesion in 1987. We could not sufficiently restore TAP1 mRNA by IFNγ (1,000 IU/ml, 3 d) treatment (data not shown). Therefore, the human TAP1 gene was delivered into melanoma cells by "shooting" the gene into the PM1-B1 cell line using the "gene gun" device Accell®. TAP1 gene expression could be monitored using a polyclonal Ab specific for the TAP-1 protein (Fig. 4). Staining of the melanoma sample 717 (1987) shows a high percentage of TAP1-positive staining tumor cells (Fig. 4 *A*). In contrast, the melanoma cell line PM1-B1 (1993) is deficient in TAP1 protein expression (Fig. 4 *B*). The single cell suspension 6129TW was similarly TAP-1 negative (data not shown). The expression of TAP-1 could be restored in ~ 20% of the PM1-B1 cells after gene gun delivery of the TAP-1 gene and selection for 10 d in G418 (Fig. 4 *C*). T cell recognition of PM1-B1 cells after TAP-1 gene transfer was examined using the polyclonal TIL line 1235 or the CTL clone A83, both specific for HLA–A2-presented peptides derived from MART-1/Melan-A. Both T cell lines recognized the positive control melanoma cell 624, but not the PM1-B1 (1993) cell line (Table III). Gene transfer of MART-1/Melan-A alone, or TAP-1 alone did not restore T cell recognition. In contrast, DFG-MART-1/Melan-A retrovirally infected PM1-B1 melanoma cells, into which the TAP-1 gene was additionally delivered by the bioballistic approach, were lysed by MART-1/Melan-A–specific TIL. The PM1-B1 cell line infected with a retroviral vector containing the HLA–A2 gene (DFG-HLA-A2) was not recognized by either effector CTL line. Based on these results, we concluded that in a melanoma resected from a patient in 1993 (compared to a lesion resected in 1987) there was (*a*) a loss of the immunodominant antigen recognized by the majority of HLA–A2-restricted and melanoma specific CD8+ CTL (MART-1/Melan-A), and (*b*) a lack of cytosolic peptide transport into the endoplasmic reticulum (ER), which is physiologically required for the assembly and transport of MHC class I molecules, as a result of impaired TAP-1 expression.

Discussion

Human melanoma cells typically express a number of "shared" antigens yielding peptide epitopes recognized by HLA–A2-restricted CD8+ cytotoxic T-lymphocytes. Such antigens are also shared among melanoma cells and their normal counterparts, melanocytes (2–10, 23–27). Each of these gene products are encoded by normal, nonmutated genes, with most of them providing several T cell epitopes, e.g., MART-1/Melan-A (three epitopes), gp100 (two epitopes), and tyrosinase (two

Table III. Simultaneous Expression of the MART-1/Melan-A Antigen with TAP-1 Restores CTL Recognition of Tumor PM1-B1 by HLA–A2-restricted and MART-1/Melan-A–specific CTL Lines TIL 1235 or CTL Clone A83

Targets	PM1-B1	PM1-B1	PM1-B1	PM1-B1	PM1-B1	Mel 624 (control)	SCS 6129 (1993)	SCS 717 (1987)
Gene transfer	—	DFG-HLA–A2	RSV-TAP1	DFG-MART-1	DFG-MART-1 + RSV-TAP-1			
Expression of								
TAP1	−	−	+	−	+	+	−	+
MART-1	−	−	−	+	+	+	−	+
Effectors:		E:T = 10:1			Percent specific Lysis			
TIL1235	0	0	1	8	27	36	0	30
TIL A83	0	0	1	6	17	27	0	20

The Mart-1/Melan-A antigen was expressed in Mel PM1-B1 cells using a retroviral vector termed DFG-MART-1/neo. TAP-1 expression was achieved using the expression plasmid RSV5-TAP1 and the gene-delivery device Accell®. After selection in G418, target cell lines were tested for CTL recognition by the HLA–A2-restricted and MART-1/Melan-A–specific TIL line 1235, or the CTL clone A83. A retroviral vector with the HLA–A2 gene (DFG-HLA–A2) served as a control. Expression of MART-1 alone showed 8 and 6% specific lysis, but simultaneous expression of both MART-1/Melan-A and TAP-1 resulted in significant CTL recognition. The HLA–A2+, MART-1/Melan-A+, Tap1+ melanoma cell line 624, as well as the SCS (freshly harvested single cell suspension) harvested from patient PM1-B1 in 1987, and in 1993 served as controls. Expression of TAP-1 in PM1-B1 did not result in significantly enhanced lysis of the autologous tumor (1993) by autologous TIL (data not shown). This observation may be due to the high frequency of MART-1/Melan-A–reactive CTL present within the autologous TIL. Such T cell effectors would not be able to recognize the MART-1/Melan-A negative, TAP-1+ PM1-B1 melanoma cell line.

epitopes) recognized by cytotoxic T cells. Recent studies revealed that 'private' tumor antigens recognized by cytotoxic T cells may result from mutated gene products; e.g., mutated CDK4 involved in cell cycle regulation (28). Such T cell–defined antigens provide excellent candidates for the development of novel tumor vaccines capable of eliciting antitumor immune responses in melanoma patients (6, 10) either in the form of native proteins or as peptide-based vaccines. Clinical trials implementing peptide-based vaccines have recently begun in the United States and in Europe.

Tumor antigen heterogeneity, expressed as quantitative differences in CTL recognition has been previously described in human melanoma, but only from a single tumor lesion and only after cloning the tumor cells in vitro (29, 30). We have described the loss of expression of a defined immunodominant antigen by a melanoma lesion in situ. Similar observations of antigen losses have been reported in murine models (31, 32) where elimination or downregulation of entire genes encoding tumor antigens was correlated with "antigen-loss" variants exhibiting decreased immunogenicity and increased tumorigenicity, i.e., in the mouse mastocytoma P815 model described by De Plaen et al. (31) and Van den Eynde et al. (32). However, in addition to the downregulation in expression of an entire tumor antigen providing peptides presented by MHC, more subtle changes may also occur. "Epitope-loss" variants induced by point mutations within a MHC class I–presented tumor antigenic peptide recognized by T cells may abrogate CTL recognition specific for the wild-type peptide (33). An additional mechanism for immune escape are point mutations within immunogenic individual peptides, which are still capable of binding to the proper MHC molecule, and still engage with the appropriate T cell receptor, but are capable of altering the quality and magnitude of the triggered T cell response (34, 35). Alternatively, point mutations within a given peptide may simply abrogate the capability of that peptide to bind to its proper MHC restriction element and hence preclude CTL reactivity (36, 37).

A striking observation in these studies is that we could demonstrate anti–MART-1/Melan-A–specific TIL from a melanoma cell, which apparently lost expression of the MART-1/Melan-A antigen. It is tempting to speculate that a vigorous antitumor CTL immune response in vivo might facilitate the generation of antigen-loss variants by eradicating tumor target cells expressing the MART-1/Melan-A antigen. Alternatively, the presence of anti–MART-1/Melan-A–directed TIL might also reflect the high immunogenicity of this particular antigen, since the T cell precursor frequency in PBL in HLA–A2+ melanoma patients, and even in normal healthy volunteers appears to be surprisingly high (38). Of note, the PM1-B1 TIL line recognized a single residual epitope (eluting in HPLC fraction 37) presented by the autologous tumor in 1987 and 1993 (Fig. 1 and Table II). This HLA–A2-associated T cell epitope appears to be shared by other HLA–A2+ melanoma cells (see Fig. 1).

Two observations appear to be of critical importance: (*a*) the immune system is capable of reacting against a "subdominant" epitope(s) (i.e., eluting in HPLC fraction 37 for PM1-B1, 1993) in the absence of the presumed immunodominant antigen presented by HLA–A2 (i.e., MART-1/Melan-A); and (*b*) this particular T cell epitope is presented in the absence of TAP-1; i.e., presumably independent of the requirement of peptide transport from the cytosol into the ER. This phenomenon appears to reflect a common feature of the HLA–A2 molecule, which has been reported to bind to leader-peptide sequences from various proteins, cleaved by proteases in the ER. Such leader-peptide sequences are independent of the active TAP-1 ATP-dependent peptide transport and represent a physiological mechanism of protein processing and "trimming" in the ER. One of the low affinity HLA–A2 binding peptides recognized by anti-melanoma–directed CTL has been reported to represent such a leader peptide cleaved during the physiologic processing of the enzyme tyrosinase in the ER (39).

Recent studies from Ferrone and Marincola (40) suggest that downregulation of TAP-1 expression is relatively frequent, at least in a proportion of cells (25–50%) within melanoma lesions. Alternatively, viral infection of human cells may lead to exclusive downregulation of TAP-1 (41, 42). TAP-independent peptide vaccines may be relevant in alternate tumor histologies where the peptide transporter molecules appear to be frequently downregulated, such as in lung (18) or cervical (19) cancer and underscore the need for individual examination of a patient's tumor lesions before immunotherapy.

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